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Immunohistochemical Analyses of Long-Term Extinction of Conditioned Fear in Adolescent Rats

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Adolescence is a period of heightened emotional reactivity and vulnerability to poor outcomes (e.g., suicide, anxiety, and depression). Recent human and animal neuroimaging studies suggest that dramatic changes in prefrontal cortical areas during adolescence are involved in these effects. The present study explored the functional implications of prefrontal cortical changes during adolescence by examining conditioned fear extinction in adolescent rats. Experiment 1 showed that preadolescent (i.e., postnatal day [P] 24), adolescent (P35), and adult (P70) rats express identical extinction acquisition following 3 white-noise conditioned stimulus (CS) and shock pairings. When tested the next day, however, adolescent rats showed almost complete failure to maintain extinction of CS-elicited freezing compared with P24 and P70 rats. It was observed in experiment 2 that following extinction, P24 and P70 rats express significantly elevated levels of phosphorylated mitogen-activated protein kinase (pMAPK) in the infralimbic cortex (IL) compared with adolescent rats. Interestingly, adolescent rats successfully exhibited long-term extinction if the amount of extinction training was doubled (experiment 3). More extinction training also led to increased phosphorylation of MAPK in the IL in these rats. These findings suggest that adolescents are less efficient in utilizing prefrontal areas, which may lead to an impairment in the maintenance of extinguished behavior.

Keywords: adolescence, extinction, fear, MAPK, mPFC

Extinction is the decrease in conditioned response to a previously trained conditioned stimulus (CS) following nonreinforced presentations of the CS (Pavlov 1927). In the past decade, extinction of conditioned fear has been studied extensively because most effective treatments for anxiety disorders rely on the process of extinction (Myers and Davis 2007; Pine et al. 2009). As a result, it is now understood that the reduction of fear as a consequence of extinction is due to new learning and expression of CS-no unconditioned stimulus (US) memory that depend on a network of neural structures such as the amygdala, hippocampus, and the medial prefrontal cortex (mPFC) (Maren and Quirk 2004; Bouton et al. 2006; Quirk and Mueller 2007; Ehrlich et al. 2009).

The mPFC, in particular, appears to be critically involved in the consolidation and the expression of extinction memory (Quirk et al. 2000; Sotres-Bayon et al. 2006). For example, permanent and temporary lesions of the ventral mPFC impair long-term extinction (e.g., Morgan et al. 1993; Sierra-Mercado et al. 2006), and neural activity in this structure predicts the strength of extinction expression (Milad and Quirk 2002). Additionally, extinction involves activation of the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) signaling pathway in the mPFC (Kim et al. 2009), and postextinction blockade of MAPK/ERK in the mPFC disrupts long-term extinction (Hugues et al. 2004, 2006). The MAPK/ERK pathway is a part of the intracellular cascade that is involved in producing long-lasting changes in synaptic efficacy (English and Sweatt 1997; Kandel 2001).

The importance of the mPFC in long-term extinction has interesting implications for extinction during adolescence. Adolescence is a critical period in development, during which the brain undergoes a dramatic reorganization involving growth of synapses and decreases in the gray matter, especially in prefrontal cortical areas (Geidd 2004; Gogtay et al. 2004; Steinberg 2005; Paus et al. 2008). For example, in a recent longitudinal study involving 375 children, adolescents, and young adults, Shaw et al. (2008) observed that the developmental trajectory of the prefrontal cortex gray matter is essentially "cubic." That is, the prefrontal cortex slowly develops to peak in volume during preadolescent childhood, then decreases dramatically during adolescence, and then increases again to stabilize by early adulthood (Shaw et al. 2008). Consistent with this finding, it has been observed that adolescents show heightened amygdala activity to emotional stimuli (e.g., fear-expressing faces) due to their inefficiency in recruiting prefrontal regions compared with preadolescent children and adults (Hare et al. 2008). Although research has provided much insight into how the changes in the brain during adolescence are involved in the heightened tolerance and approach behavior to rewarding stimuli (Spear 2000; Steinberg 2005; Eshel et al. 2007), our understanding of the consequences of the prefrontal changes during adolescence on aversive learning, especially fear extinction, is very limited. It may be the case that extinction of conditioned fear is different in adolescence due to the changes occurring in the mPFC during adolescence. Therefore, the present study examined extinction of conditioned fear in adolescence. In experiment 1, we examined renewal of extinguished fear in P24 (preadolescent), P35 (adolescent), and P70 (adult) rats. Follow-up experiments further explored extinction in adolescent rats by examining postextinction phosphorylation of MAPK in the mPFC using immunohistochemistry.

Materials and Methods

Subjects

Experimentally naive Sprague-Dawley derived male rats were bred and housed in the School of Psychology, University of New South Wales. Before weaning, rats were housed with their littermates and mother in plastic boxes covered by a wire lid. At P21 (±1), male rats from 2 or more litters were weaned and housed in groups of 8 and maintained on a 12-h light/dark cycle (lights on at 6 AM) with food and water available ad libitum. All animals were treated according to the

principles of animal use outlined in The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition), and all procedures were approved by the Animal Care and Ethics Committee at the University of New South Wales.

Apparatus

Two types of experimental chambers were used to provide different contexts. Context A was rectangular (20 cm long × 12 cm wide × 12 cm high), with the front wall, rear wall, and ceiling constructed of clear Plexiglas. The floor and side walls consisted of stainless steel rods set 1.3 cm apart. Two high-frequency speakers were located 8 cm from either side of the chamber. A custom-built constant-current shock generator could deliver electric shock to the floor of the chamber as required. A tray of bedding was placed 7 cm below the grid floor. This chamber was housed within a separate wood cabinet with an infrared light and a white light-emitting diode (LED). Context B was rectangular (30 cm long × 30 cm wide × 23 cm high) and wholly constructed of Plexiglas, with the exception of the grid floor, which was the same as in the first set of chambers. A clear Plexiglas sheet (35 × 35 cm) was placed beneath the grid floor. All the walls were transparent, except for two side walls that consisted of vertical black and white stripes (5 cm each). Two high-frequency speakers were mounted on the ceiling of each of these chambers. This chamber was housed within a separate wood cabinet with an infrared light and a white LED. All training occurred in context A and extinction occurred in context B. All test sessions occurred in context B, except in experiment 1 where half of the rats were tested in context A (different to extinction) and the other half were tested in context B (same as extinction).

The CS was a white noise; noise level was increased by 8 dB when the CS was presented. A computer controlled all presentations of the CS and the footshock US. The software and hardware used were developed at the University of New South Wales.

Procedures

Handling and Preexposure

All rats were handled for 2 days for 3 min per day prior to the beginning of the experiment. After each session of handling, rats were exposed to context A for 8 min to reduce baseline freezing to context A. This was done to reduce discrepancy in baseline freezing at test between ABA and ABB groups in experiment 1; to maintain consistency, rats in experiments 2 and 3 also received preexposure sessions.

Training

Rats were placed in an experimental chamber, and after a 2-min adaptation period, the CS was presented for 10 s. The shock US (0.6 mA, 1 s) was administered during the last second of the CS. All rats received 3 pairings of the CS and US. The intertrial interval (ITI) ranged from 85 to 135 s with a mean of 110 s.

Extinction

The extinction session commenced 24 h after training. After a 2-min adaptation period, the 10-s CS was presented with a 10-s ITI. In experiments 1 and 2, extinction training consisted of the presentation of 30 CSs in the absence of shock. In experiment 3, rats were presented with either 30 or 60 CSs in the absence of shock. For statistical analyses of within-session extinction, 6 CS presentations were always averaged to represent 1 block of extinction.

Testing

Rats were placed in an experimental chamber, and their baseline level of freezing in the absence of the CS was recorded for 1 min. The CS was then presented, and freezing was recorded for 2 min. Freezing was scored by a time sampling procedure whereby each rat was scored every 3 s as freezing or not freezing by an observer who was blind to the group assignment of each rat. Freezing was defined as the absence of all movement other than that required for respiration.

Baseline Criterion for CS Tests and Statistical Analyses

CS-elicited freezing is difficult to detect if rats display high baseline levels of freezing. Therefore, a baseline criterion was introduced. Specifically, if a rat was freezing more than 50% of the pre-CS period at test, it was not tested for CS-elicited freezing. In experiment 1, 2 rats each from groups P24-Same and P24-Different and 1 rat each from groups P35-Same, P35-Different, and P70-Same were not tested due to >50% baseline freezing. In experiment 3, 1 rat from group Single was not tested due to >50% baseline freezing.

Tissue Processing, Immunohistochemistry, and Neuronal Counting

One hour after extinction, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused with 50 ml of 0.9% saline, containing 1% sodium nitrite and heparin (5000 international units/ml), followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were postfixed for 1 h in the same fixative and placed in 20% sucrose solution overnight. Brains were blocked using a matrix (Stoelting) aligned to the rat brain atlas (Paxinos and Watson 1998) and 40-μm coronal sections were cut using a cryostat (Microm HM 560) in 4 serially adjacent sets and stored in 0.1% sodium azide in 0.1 M PB saline (pH 7.2). Series of sections were selected from each rat, and immunohistochemistry was used to reveal phospho-p44-42MAPK. Free floating sections were washed repeatedly in 0.1M PB (pH 7.4) followed by two 30-min washes in 50% ethanol, the second of which contained 3% H_2O_2, and were then incubated in 5% normal horse serum (NHS) in PB (pH 7.4) for 30 min. Sections were then incubated in rabbit antiserum against pMAPK (1:1000; phospho-p44-42MAPK [Thr202/Tyr204] Rabbit mAb; Cell Signaling Technology) for 48 h at 4°C, with gentle agitation. The primary antibody was diluted in PBT-X which consisted of 0.1 M PB (pH 7.4) containing 2% NHS and 0.2% Triton X-100. After washing off unbound primary antibody, sections were incubated overnight at room temperature in biotinylated donkey anti-rabbit IgG (1:1000; Jackson Immunoresearch Laboratories) diluted in PBT-X. After washing, sections were then incubated for 2 h at room temperature in ABC reagent (Vector Elite kit: 6 μl/ml avidin and 6 μl/ml biotin; Vector Laboratories). Black immunoreactive (IR) cytoplasm labeled for pMAPK was revealed by a nickel-intensified diaminobenzidine (DAB) reaction, with peroxide being generated by glucose oxidase. In this DAB reaction, sections were washed in PB (pH 7.4), followed by 0.1 M acetate buffer (pH 6.0), and then incubated for 15 min in 0.1 M acetate buffer (pH 6.0) containing 2% nickel sulfate, 0.025% 3,3 DAB, 0.04% ammonium chloride, and 0.02% D-glucose. The peroxidase reaction was started by adding 0.2 μl/ml glucose oxidase and stopped using acetate buffer (pH 6.0). Brain sections were then washed in PB (pH 7.4). Sections were mounted onto gelatin-treated slides, dehydrated, cleared in xylene, and coverslipped with DPX (Biolab).

Neuronal counting.

The quantification of phospho-MAPK immunoreactivity was carried out at ×10 magnification using manual blind counts of neurons using grid eyepieces. All sections counted were 160 μm apart. The brain region analyzed was mPFC over 3 sections (3.20–2.88 mm anterior to Bregma). In each rat, pMAPK-IR was quantified within predefined boundaries delineating each neural structure according to the rat brain atlas (Paxinos and Watson 1998). The top border of the prelimbic cortex (PrL) was identified by aligning the counting grid with the top of the corpus callosum (2.5 mm ventral to Bregma). The size of the areas examined in this study was kept constant across all ages and experiments (1 × 1 mm for PrL and 1 × 0.7 mm for infralimbic cortex [IL]).

Results

Adolescent Rats Fail to Maintain Long-Term Extinction 24 h after Extinction Training

Experiment 1 examined whether there are behavioral differences in fear extinction across development in the rat. P24,

P35, and P70 rats were conditioned with 3 noise-shock pairings and 1 day later, all received 30 nonreinforced CS presentations. All rats were tested the next day for CS-elicited freezing either in same or different context to where extinction occurred.

Pavlovian fear conditioning and extinction proceeded similarly across different ages and conditions (Fig. 1*A,B*). A mixed-design ANOVA of the CS-elicited freezing data during conditioning yielded a significant main effect of Conditioning Trial ($F_{2,118}$ = 110.7, P < 0.0001). There were no effects of Age or Test Context, and there were no significant interactions (Fs < 1). There were also no differences in preextinction level of freezing across age ($F_{2,118}$ = 2.93, P = 0.06; see Table 1). For analyses of within-session extinction, CS-elicited freezing data were collapsed into blocks, each block consisting of 6 CS presentations (i.e., 30 CS presentations = 5 blocks). A mixed-design ANOVA of the extinction data yielded a significant main effect of Block ($F_{4,236}$ = 90.4, P < 0.0001). There were no effects of Age ($F_{2,59}$ = 1.4, P = 0.26) or Test Context ($F_{1,59}$ = 1.0, P = 0.32), and there were no significant interactions (Fs < 1). As there was no effect of test context, Figures 1*A,B* show CS-elicited freezing during conditioning and extinction collapsed across the test context condition.

The mean levels of CS-elicited freezing at test are shown in Figure 1*C*. ANOVA did not reveal any significant group differences in baseline freezing levels at test (see Table 2). During the CS presentation, rats across all age groups exhibited renewal of extinguished freezing when tested in a context different from where extinction training occurred. ANOVA of the CS-elicited freezing data revealed main effects of Age ($F_{2,59}$ = 5.7, P < 0.01), Test Context ($F_{1,59}$ = 42.6, P < 0.0001), and a Age × Test Context interaction ($F_{2,59}$ = 8.7, P < 0.0001). To understand the significant interaction, post hoc Student–Newman–Keuls (SNK) multiple pairwise comparisons were done for each test context condition. When tested in a different context as extinction training, CS-elicited freezing did not differ across different ages (Ps > 0.3). When tested in the same context as extinction training, P35 rats showed significantly higher CS-elicited freezing compared with P24 and P70 rats (P < 0.05 and P < 0001, respectively). We specifically examined whether rats exhibited long-term extinction by comparing CS-elicited freezing levels during block 1 of extinction and at test for each age. Paired t-tests showed that P24 and P70 rats froze significantly less during test compared with during block 1 of extinction (P < 0.001 and P < 0.0001, respectively). However, CS-elicited freezing during block 1 of extinction and test did not differ in P35 rats (P = 0.15).

Taken together, it was observed that P24, P35, and P70 rats show similar fear acquisition and within-session extinction using the present parameters. At test the next day, however, P35 rats failed to maintain extinction of CS-elicited freezing.

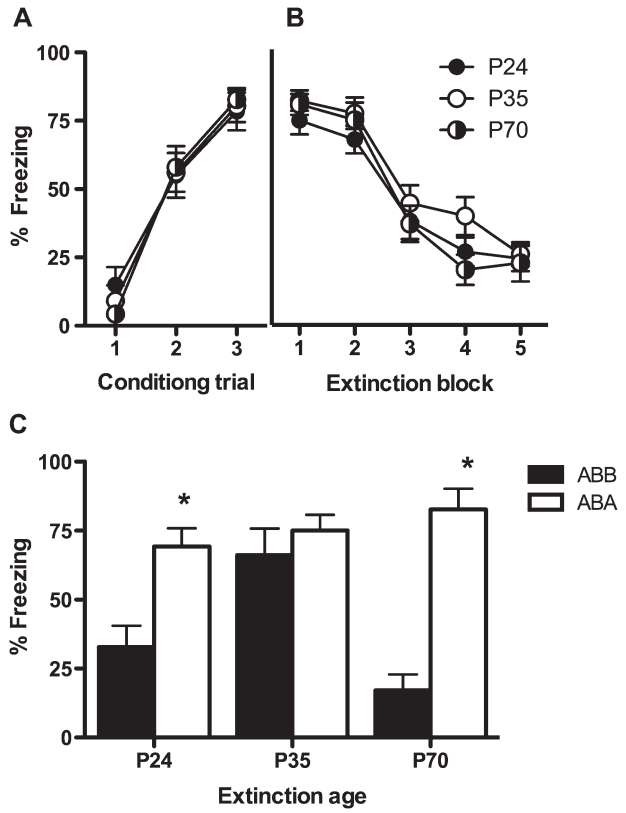

Figure 1. The mean (±standard error of the mean) levels of CS-elicited freezing during (*A*) conditioning and (*B*) extinction collapsed across test context condition in experiment 1. Rats of all ages displayed comparable levels of CS-elicited freezing during conditioning and extinction. (*C*) The mean (±SEM) CS-elicited freezing at test. P24 and P70 rats showed renewal of extinguished fear when tested in a different context to extinction. In contrast, P35 rats failed to show extinguished fear regardless of the test context. P24-ABB (n = 10), P24-ABA (n = 10), P35-ABB (n = 11), P35-ABA (n = 11), P70-ABB (n = 12), and P70-ABA (n = 11). "*" Indicates a significant difference to group Same in the matching age condition (Ps < 0.001).

Following Extinction, Adolescent Rats Show Less Phosphorylation of MAPK in the IL Compared with Preadolescent and Adult Rats

Experiment 2 examined whether the developmental differences observed in long-term extinction in experiment 1 were due to differences in postextinction mPFC MAPK phosphorylation. Rats received conditioning and extinction as described

Table 1
Mean (±) SEM % freezing at baseline before extinction

Experiment	Groups	Mean % (±SEM) freezing
1	P24	21 (±5)
	P35	23 (±5)
	P70	9 (±4)
2	P24	22 (±11)
	P35	12 (±4)
	P70	10 (±9)
3	Single	19 (±5)
	Double	13 (±6)

Note: There were no significant group differences in any experiment.

Table 2
Mean (±) SEM % freezing at baseline before test

Experiment	Groups	Mean % (±SEM) freezing
1	P24-Same	18 (±6)
	P24-Different	17 (±6)
	P35-Same	10 (±5)
	P35-Different	13 (±5)
	P70-Same	5 (±2)
	P70-Different	10 (±4)
3	None	7 (±2)
	Single	15 (±3)
	Double	3 (±1)

Note: There were no significant group differences in either experiment.

in experiment 1. A "no extinction" control group (which consisted of P24, P35, and P70 rats) was conditioned and then exposed to the extinction context without any CS presentations. One hour following the extinction/exposure session, rats were perfused and the phosphorylation of MAPK in the mPFC was assessed by immunohistochemistry.

Consistent with experiment 1, conditioning and extinction proceeded similarly across the different age groups (Fig. 2*A*,*B*). Additionally, there were no significant age differences in CS-elicited freezing across conditioning trials in No Extinction rats ($F < 1$); therefore, data from these rats were collapsed into a single No Extinction group for subsequent statistical analyses. A mixed-design ANOVA of the conditioning data yielded a significant main effect of Conditioning Trial ($F_{2,44} = 56.9$, $P < 0.0001$). The effect of Group and Trial × Group interaction was not significant (Fs < 1). There were also no differences in preextinction level of freezing across age ($F < 1$). A mixed-design analysis ANOVA of the extinction data yielded a significant main effect of Block ($F_{4,64} = 27.8$, $P < 0.0001$). The effect of Group and Block × Group interaction was not significant (Fs < 1).

Figures 2*C*,*D* present the number of pMAPK-IR neurons observed in the prelimbic and infralimbic cortices of the mPFC, respectively. One-way ANOVA showed that there were no significant age differences in pMAPK-IR neuron counts in No Extinction rats for both PrL and IL (Fs < 1), therefore, data from these rats were collapsed into No Extinction groups in PrL and IL for subsequent statistical analyses. One-way ANOVA indicated that there were no significant group differences in the number of pMAPK-IR neurons in the PrL ($F_{3,25} = 1.5$, $P = 0.24$). In contrast, one-way ANOVA of IL data showed that there are significant group differences ($F_{3,25} = 4.4$, $P < 0.05$). Post hoc SNK multiple pairwise comparisons showed that P24 and P70 rats exhibit significantly elevated phosphorylation of MAPK in the IL compared with P35 rats and to the No Extinction control group (Ps < 0.05). The P35 and No Extinction groups were not different from each other ($P = 0.99$). It appears that extinction in P24 and P70 rats leads to phosphorylation of MAPK in the IL compared with rats that do not receive extinction. In contrast, P35 rats display comparable pMAPK-IR neurons in the IL to rats that did not receive any extinction using the present parameters. Photomicrographs of the representative immunostained neurons in the IL are shown in Figures 3*A*,*B*.

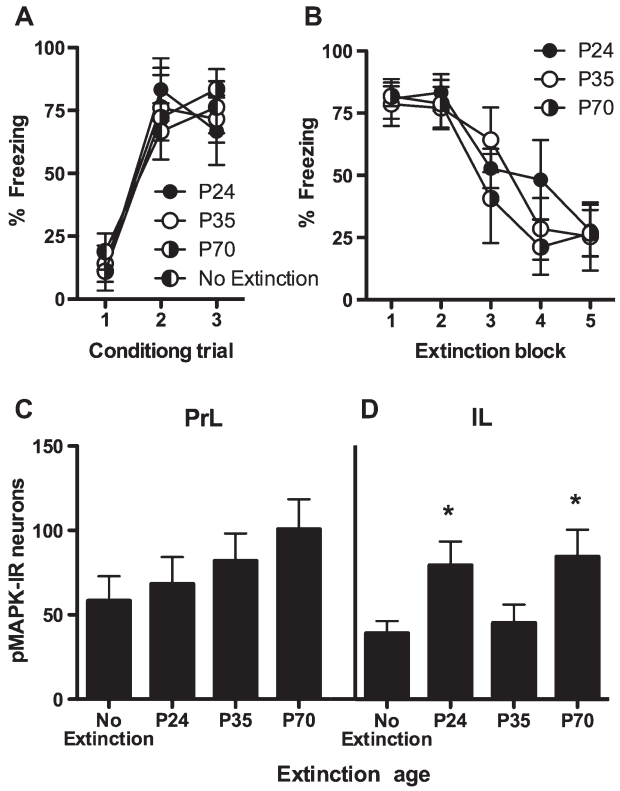

Figure 2. The mean (±SEM) levels of CS-elicited freezing during (*A*) conditioning and (*B*) extinction in experiment 2. Rats of all ages displayed comparable levels of CS-elicited freezing during conditioning and extinction. (*C*) Numbers of pMAPK-IR neurons in the PrL and (*D*) IL following extinction training in experiment 2. Extinction of conditioned fear led to an elevated number of pMAPK-IR neurons in IL in P24 and P70 rats but not in P35 rats. P24 ($n = 6$), P35 ($n = 7$), P70 ($n = 6$), and No Extinction ($n = 7$). "*" Indicates a significant difference to groups No Extinction and P35 (Ps < 0.05).

More Extinction Training in Adolescent Rats Leads to Increased Phosphorylation of MAPK in the mPFC and Facilitation of Long-Term Extinction

In experiment 1, adolescent rats failed to maintain extinguished fear response 1 day following extinction compared with P24 and P70 rats. Experiment 2 then showed that P35 rats, compared with P24 and P70 rats, show significantly lower phosphorylation of MAPK in IL following extinction. Experiment 3 investigated whether giving more extinction trials attenuates the extinction impairment exhibited by P35 rats. It may be the case that the difference observed in extinction across adolescence is a quantitative difference—P35 rats may simply need more extinction training to express similar amounts of extinction as P24 and P70 rats. This hypothesis is consistent with the suggestion that similar neural processes are employed by adolescents as adults do in cognitive tasks but adolescents are just less efficient in utilizing the mPFC (Luna and Sweeney 2005; Steinberg 2005; Hare et al. 2008). Alternatively, P35 rats may be qualitatively different from P24 and P70 rats in the neural processes of extinction (i.e., the mPFC may not be involved in extinction during adolescence) and giving more extinction training may have no impact at this age. Recent studies in our laboratory have shown that the IL is not critical for extinction in preweanling aged (i.e., P17) rats, and as a consequence, it appears that extinction is qualitatively different in P17 rats (Kim et al. 2009; Kim and Richardson 2010). In the present experiment, P35 rats were conditioned and then received either 30 or 60 nonreinforced CS presentations during a single session. A "none" control group was exposed to the extinction context without any CS presentations. After extinction, half of the rats in each group were perfused to examine phosphorylation of MAPK in the mPFC. The remaining half from each groups were tested for CS-elicited freezing the next day.

There were no differences in fear conditioning across groups (Fig. 4*A*). A mixed-design ANOVA of the conditioning data yielded a significant main effect of Conditioning Trial ($F_{2,88} = 123.1$, $P < 0.0001$). The effect of Group and Trial × Group interaction was not significant (Fs < 1). There were also no differences in preextinction level of freezing across extinction groups ($t < 1$) (Table 1). A mixed-design ANOVA of the first 5 blocks of extinction data yielded a significant main effect of Block ($F_{4,116} = 31.5$, $P < 0.0001$) but no effects of

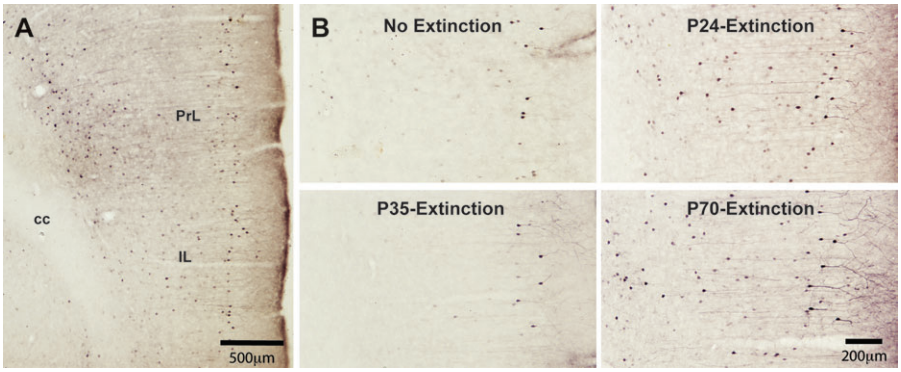

Figure 3. (*A*) Photomicrograph of the mPFC. (*B*) Photomicrograph of pMAPK-IR neuronal staining in the IL in experiment 2. cc, corpus callosum.

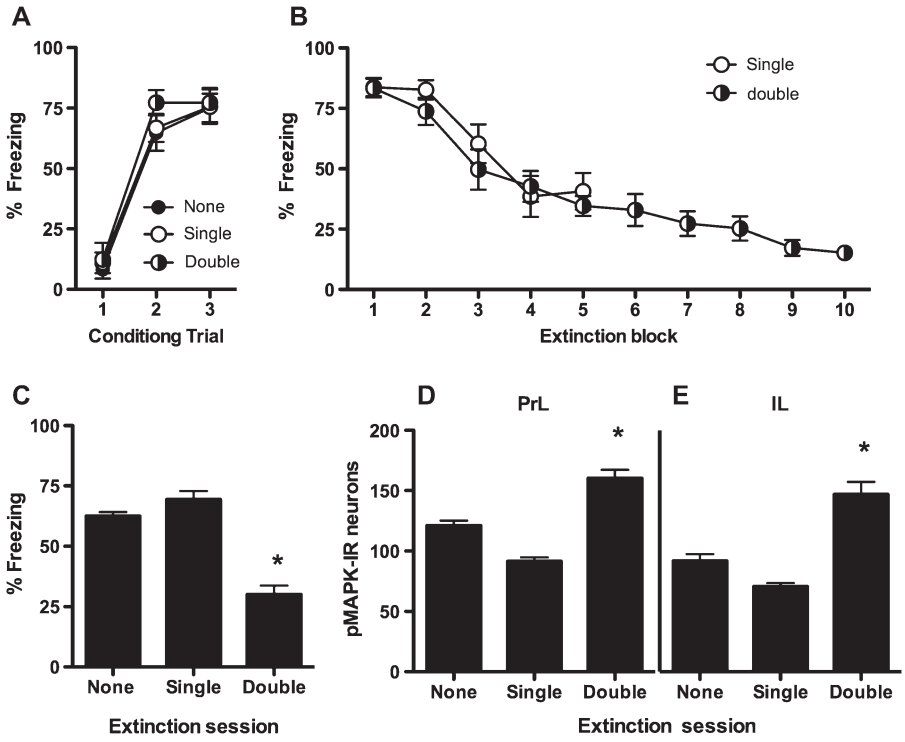

Figure 4. The mean (±standard error of the mean) levels of CS-elicited freezing during (*A*) conditioning and (*B*) extinction in experiment 3. (*C*) The mean (±SEM) CS-elicited freezing at test. Giving double the amount of extinction in P35 rats significantly facilitated long-term extinction. None ($n = 9$), Single ($n = 8$), and Double ($n = 9$). (*D*) Numbers of pMAPK-IR neurons in the PrL and (*E*) IL following extinction training in experiment 3. Double the amount of extinction training in P35 rats significantly increased phosphorylation of MAPK following extinction. None ($n = 7$), Single ($n = 7$), and Double ($n = 7$). " *" Indicates a significant difference to all the other groups (Ps < 0.05).

Group or interactions (Fs < 1). A repeated-measures ANOVA of the last 5 blocks in group Double showed a significant effect of Block ($F_{4,60} = 4.0$, $P < 0.01$); that is, the fear continued to extinguish across these additional trials in this group.

There were no differences in baseline levels of freezing at test (Table 2). In contrast, one-way ANOVA showed that there were significant group differences in CS-elicited freezing at test ($F_{2,23} = 7.1$, $P < 0.005$). SNK post hoc multiple pairwise comparisons revealed that P35 rats that received double the number of CS presentations (i.e., 10 blocks) during extinction froze significantly less than the rats that received single (i.e., 5 blocks) or none (Ps < 0.05). There was no significant difference between groups None and Single ($F < 1$). This result replicates experiment 1 in that giving 5 blocks of extinction is sufficient for P35 rats to exhibit within-session extinction learning, but long-term extinction is impaired in these rats when tested 24 h later. Interestingly, giving double the amount of CS presentations led to long-term extinction in these rats, indicating that adolescent rats are able to maintain extinguished behavior if given enough extinction training. It appears that the developmental difference in extinction observed in adolescence is quantitative. That is, similar processes are likely to be involved in extinction in P24, P35, and P70 rats; however, P35 rats need more extinction trials to maintain the acquired extinguished response compared with P24 and P70 rats.

Immunohistochemical data were consistent with the behavioral data observed at test (Fig. 4*D*,*E*). Specifically, postextinction phosphorylation of MAPK in the mPFC was substantially higher in P35 rats that received double the amount of extinction training compared with the other groups. One-way

ANOVA showed that there were significant group differences in both PrL ($F_{2,18} = 9.0$, $P < 0.005$) and IL ($F_{2,18} = 6.1$, $P < 0.01$). SNK post hoc tests showed that P35 rats that received double the amount of extinction trials expressed significantly more pMAPK-IR neurons in the PrL and IL than the other groups (Ps < 0.5). These results suggest that giving extra extinction trials facilitate phosphorylation of MAPK in the mPFC, which appears to be then involved in the maintenance of extinction in adolescent rats.

Discussion

The present study explored fear extinction using behavioral and immunohistochemical methods in P24, P35, and P70 rats. In experiment 1, P35 rats showed significant impairment in long-term extinction compared with P24 and P70 rats despite displaying comparable fear acquisition and within-session extinction. Experiment 2 extended this finding by demonstrating that P24 and P70 rats express significantly elevated levels of pMAPK in the IL following extinction compared with P35 rats. In fact, P35 rats showed a comparable number of pMAPK-IR neurons in the IL as rats that did not receive any extinction training. It was then observed in experiment 3 that P35 can show long-term extinction if given double the amount of extinction training. More extinction training also led to increased phosphorylation of MAPK in the PrL and IL in rats this age. These findings suggest that extinction processes in adolescent rats are quantitatively different from preadolescent and adult rats due to the attenuated postextinction neural activity in the mPFC.

To our knowledge, there have been 2 studies directly examining extinction in adolescent animals. Hefner and Holmes (2007) examined differences between preadolescent (postnatal day [P] 28), adolescent (P42), and early adult (P56) mice in conditioned fear acquisition and extinction. In that study, no differences were found between adolescent and early adult mice in fear acquisition and extinction. However, extinction retention was not tested the next day. In another study, Brenhouse and Anderson (2008) examined extinction and reinstatement of cocaine-induced conditioned place preference in adolescent (P38) and adult (P77) rats. It was demonstrated that adolescent rats took many more days to extinguish conditioned place preference and showed a stronger reinstatement effect when given a US reminder. However, the results of that study were confounded by age differences in initial conditioned preference strength (i.e., adolescent rats showed stronger conditioned place preference throughout the experiment). It is clear that further studies are necessary to examine potential developmental differences in extinction during adolescence.

Fear Extinction in Adolescent Rats

In the present study, adolescent rats were impaired in maintaining extinguished fear compared with preadolescent and adult rats (experiments 1 and 3). This result may be due to the rapid changes that occur in the mPFC during adolescence. Adolescent changes in the PFC involve rapid myelinization and growth of afferent/efferent axons and dendrites, maturation and reorganization of synapses, and changes in neurochemistry such as dopaminergic and inhibitory γ-aminobutryic acidergic (GABAergic) activity (Cunningham et al. 2002, 2008; Tseng and O'Donnell 2007; Tseng et al. 2008). Coupled with reported decreases in prefrontal gray matter (Geidd 2004; Gogtay et al. 2004; Steinberg 2005; Markham et al. 2007; Paus et al. 2008; Shaw et al. 2008), the results of the present study may be due to adolescent rats having impaired efficiency in utilizing the mPFC in fear extinction. This interpretation is supported by experiment 2 in which adolescent rats showed significantly less phosphorylation of MAPK in the IL compared with preadolescent and adult rats following extinction.

Impaired efficiency of the mPFC during adolescence is also consistent with the few existing functional magnetic resonance (fMRI) studies on the effects of exposure to fearful faces in adolescent humans (Monk et al. 2003; Luna and Sweeney 2005; Hare et al. 2008). For example, Monk et al. (2003) demonstrated that when healthy adolescents and adults were focusing on subjective emotions while attending to fearful faces, adolescents showed less activation of prefrontal areas and more activation of the amygdala. Additionally, clinically anxious adolescents consistently display heightened activity in the amygdala to emotional faces compared with healthy adolescents, and it was suggested that those findings were due to compromised PFC function (Monk et al. 2008; Beesdo et al. 2009). However, it is yet to be established that reduced PFC function is indeed causal to the different amygdala reactions in adolescents humans described above. Recent studies in rodents indicate that the amygdala itself undergoes substantial changes across adolescence (Pan et al. 2009; Rubinow and Juraska 2009). In particular, Rubinow and Juraska (2009) observed that the basolateral amygdala (BLA) volume increases between P20 and P35 and then decreases between P35 and P90 (this finding inversely mirrors the volume changes in the PFC in humans). Furthermore, the BLA sends many projections to the mPFC, and it has been proposed that the dramatic "infiltration" of BLA afferents to the ventral mPFC GABAergic inhibitory interneurons during adolescence is responsible for the adolescent onset of psychiatric disorders (Cunningham et al. 2002, 2008). Therefore, it cannot be ruled out that the findings of the present study are due to the amygdala's effects on the mPFC in adolescent rats. However, it was observed in the present study that adolescent rats showed extinction retention deficits despite displaying comparable fear acquisition and within-session extinction, a result that is also consistent with a previous study on fear extinction in adolescence (Hefner and Holmes 2007). As fear acquisition and within-session extinction processes rely heavily on the amygdala, the present pattern of results suggests that the amygdala may not be an important site of change in extinction across development.

Maturation, rather than Development, of the Brain during Adolescence

Interestingly, the present study demonstrated that the impairment in maintaining extinction in adolescent rats could be overcome if rats of this age receive double the amount of extinction training (experiment 3). This effect was coupled with enhanced phosphorylation of MAPK in the mPFC in these rats. These results indicate that the adolescent difference in extinction observed in experiments 1 and 2 is quantitative. That is, the same neural mechanisms are involved in extinction during adolescence, but the adolescent rats are impaired in maintaining the extinguished fear responses and require more extinction training compared with preadolescent and adult rats. This idea is consistent with previous neuroimaging and/or

cognitive control studies showing that adolescents can display adult-like behavior, but they require repeated training and need to recruit prefrontal regions more than adults (Luna and Sweeney 2005; Steinberg 2005; Hare et al. 2008).

Taken together, the quantitative changes during adolescence observed in the present study as well as in others is well captured by the idea that adolescence is not a period of distinct development of the prefrontal cortex but rather is a period of "maturation"—that is, the prefrontal cortex has a similar role in adolescence as adulthood, but it is impaired in capacity, speed, and efficiency during adolescence (Luna and Sweeney 2005; Steinberg 2005). Specifically, Luna and Sweeney (2005) suggested that developmental changes in cognition during adolescence are distinct from those that occur earlier in infancy and childhood as adolescent changes involve improvements in existing capacities rather than the acquisition of new abilities. Indeed, the quantitative difference observed in extinction during adolescence contrasts with the qualitative difference in extinction displayed by infant rats—P17 rats appear to rely on a fundamentally different mechanism, such as erasure, during extinction (Kim and Richardson 2008, 2010). For example, P17 rats do not exhibit renewal or reinstatement, whereas P24 rats do (Kim and Richardson 2007a, 2007b; Yap and Richardson 2007). Further, it was recently demonstrated that P16 mice do not show spontaneous recovery of extinguished fear when tested 7–8 days after extinction training, whereas P23 mice do (Gogolla et al. 2009). It should be noted that P24 rats maintaining low levels of freezing 24 h after extinction training in the present study is not inconsistent with Gogolla et al. (2009) because, in that study, P23 mice also did not show any spontaneous recovery when tested 24 h after extinction training. Also, extinction in adult and P24 rats, but not in P17 rats, is GABA- and *N*-methyl-D-aspartate dependent (Harris and Westbrook 1998; Santini et al. 2001; Kim and Richardson 2007a; Langton et al. 2007). Finally, the mPFC is critical for long-term extinction in P24 and adult rats, but not in P17 rats (Sierra-Mercado et al. 2006; Kim et al. 2009). Interestingly, it has been suggested that the neural mechanisms underlying extinction in P24 and adult rats are similar (Kim and Richardson 2009), and indeed, no behavioral or neural differences were found between P24 and P70 rats in the present study. Therefore, changes in the neural circuitry underlying extinction up to 24 days of age could be described as "development" while changes from P24 to adulthood could be described as "maturation."

The results of the present study suggest that the extinction system undergoes transition from functional (e.g., P24) to nonfunctional (e.g., P35) to functional (e.g., in adulthood) with age. It is yet unclear which biological mechanisms are responsible for such transition. As mentioned earlier, it may be the case that the sudden increase in BLA afferents to the ventral mPFC GABAergic interneurons destabilize and impair mPFC function (Cunningham et al. 2002, 2008). More importantly, adolescence is a marked period for sexual maturation; therefore, it is likely that gonadal hormones are critically involved in the observed impaired long-term extinction during adolescence. Interestingly, Sisk and her colleagues have observed that preadolescent hamsters are able to exhibit mature sexual behavior for reproduction if primed with testosterone, indicating that the reproductive system is functional before adolescence (Schulz et al. 2004, 2006). However, they observed that the dramatic increase in hormones during adolescence was a necessary period to mature and refine the existing reproductive system (Schulz et al. 2004, 2006). It would be interesting to examine in future how hormonal changes during development influence cognitive functions across development.

The mPFC and Fear Extinction

The present results have interesting implications for the role of the mPFC in fear extinction. Specifically, only P35 rats failed to show long-term extinction although rats of all ages displayed similar within-session extinction. This result was followed by a significantly reduced number of postextinction pMAPK-IR neurons in the IL in P35 rats compared with P24 and P70 rats. These results suggest that phosphorylation of MAPK in the IL is critical for consolidation of extinction and implies that the IL is not involved in the acquisition of extinction. This implication is consistent with previous studies that suggest that IL is critical for extinction consolidation rather than acquisition of extinction (Quirk et al. 2000; Milad and Quirk 2002; Hugues et al. 2004, 2006; Sierra-Mercado et al. 2006).

However, increased extinction training led to increased IL pMAPK-IR neurons and successful long-term extinction in P35 rats in the present study (experiment 3). This result suggests that the IL is engaged during extinction acquisition as prolonged within-session extinction significantly elevated phosphorylation of MAPK in IL. Such finding is consistent with a recent study that examined the "immediate extinction deficit" (IED) effect (Kim et al. 2010). IED refers to the observation that extinction training administered shortly after the acquisition session does not effectively induce long-term extinction (Maren and Chang 2006). In Kim et al. (2010), it was observed that rats that received immediate extinction failed to show long-term extinction despite exhibiting comparable within-session extinction to delayed extinction rats. However, when rats received microstimulation of the IL paired with CS presentations during immediate extinction training, long-term extinction was observed the next day. Therefore, both Kim et al. (2010) and the present study suggest that the IL needs to be substantially engaged during extinction acquisition, either by electrical stimulation or extended extinction training, in order to maintain its plasticity and successfully consolidate the extinction memory. That is, although the IL might not be critical for extinction acquisition per se, IL activation during extinction acquisition may be critical for subsequent extinction consolidation.

It should be noted that the increase in phosphorylation of MAPK following the extended extinction training was not only observed in the IL but also in the PrL in adolescent rats (experiment 3). This finding is inconsistent with experiment 2 and previous reports on the PrL not being significantly involved in extinction (Kim et al. 2009; Laurent and Westbrook 2009). It may be the case that the PrL is increasingly involved in extinction with increasing extinction trials. Alternatively, increased overall amount of CS-elicited retrieval of memory in P35 rats during the extended extinction training may have triggered lasting synaptic changes in the PrL. That is, we have observed in another study that 15 min following memory retrieval, there is an increase in the phosphorylation of MAPK in both the PrL and IL; however, this increase was only maintained in the PrL but not in the IL 1 h postretrieval (JH Kim, S Li, A Hamlin, G McNally, and R Richardson, in

preparation). All rats were perfused 1 h postextinction in the present study; therefore, the increased number of pMAPK-IR neurons in the PrL observed in experiment 3 may be a PrL-specific effect due to memory retrieval. Further studies are necessary to address this issue.

Overall, the present study and others on fear extinction during development portray a nonmonotonic function across life until adulthood. That is, there is a "developmental lull" in proficient extinction during adolescence, as opposed to a more linear progressive emergence as a function of age. Considering that the onset of most psychiatric disorders is before adulthood (Newman et al. 1996; Kessler et al. 2005), it appears that more attention needs to be given to examining extinction during development, in both clinical and preclinical settings. Many neural mechanisms underlying adolescent behavior show a great amount of conservation across species (Spear 2000, 2004), but it is yet unknown whether extinction mechanisms observed across developing rats is conserved in humans. If so, then the present findings suggest that adolescent humans may be impaired in maintaining extinguished fear, therefore, need more attention and support in the treatment of their anxiety disorders.

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